Applied and Environmental Microbiology, Aug. 2010, p. 5105–5112 Vol. 76, No. 15
0099-2240/10/$12.00 doi:10.1128/AEM.00183-10

Detection of Microcystin-Producing Cyanobacteria in Missisquoi Bay, Quebec, Canada, Using Quantitative PCR▽

Nathalie Fortin,[1] Rocio Aranda-Rodriguez,[2] Hongmei Jing,[4] Frances Pick,[3] David Bird,[4] and Charles W. Greer[1]*

National Research Council Canada, Biotechnology Research Institute, Montreal, QC, Canada[1]; Environmental Health Center, Health Canada, Ottawa, ON, Canada[2]; Biology Department, University of Ottawa, Ottawa, ON, Canada[3]; and Biological Sciences, University of Quebec at Montreal, Montreal, QC, Canada[4]

Received 25 January 2010/Accepted 8 June 2010

Toxic cyanobacterial blooms, as well as their increasing global occurrence, pose a serious threat to public health, domestic animals, and livestock. In Missisquoi Bay, Lake Champlain, public health advisories have been issued from 2001 to 2009, and local microcystin concentrations found in the lake water regularly exceeded the Canadian drinking water guideline of 1.5 μg liter^{-1}. A quantitative PCR (Q-PCR) approach was developed for the detection of blooms formed by microcystin-producing cyanobacteria. Primers were designed for the β-ketoacyl synthase (*mcyD*$_{KS}$) and the first dehydratase domain (*mcyD*$_{DH}$) of the *mcyD* gene, involved in microcystin synthesis. The Q-PCR method was used to track the toxigenic cyanobacteria in Missisquoi Bay during the summers of 2006 and 2007. Two toxic bloom events were detected in 2006: more than 6.5 × 10^4 copies of the *mcyD*$_{KS}$ gene ml^{-1} were detected in August, and an average of 4.0 × 10^4 copies ml^{-1} were detected in September, when microcystin concentrations were more than 4 μg liter^{-1} and approximately 2 μg liter^{-1}, respectively. Gene copy numbers and total microcystin concentrations (determined by enzyme-linked immunosorbent assay [ELISA]) were highly correlated in the littoral ($r = 0.93$, $P < 0.001$) and the pelagic station ($r = 0.87$, $P < 0.001$) in 2006. In contrast to the situation in 2006, a cyanobacterial bloom occurred only in late summer-early fall of 2007, reaching only 3 × 10^2 *mcyD*$_{KS}$ copies ml^{-1}, while the microcystin concentration was barely detectable. The Q-PCR method allowed the detection of microcystin-producing cyanobacteria when toxins and toxigenic cyanobacterial abundance were still below the limit of detection by high-pressure liquid chromatography (HPLC) and microscopy. Toxin gene copy numbers grew exponentially at a steady rate over a period of 7 weeks. Onshore winds selected for cells with a higher cell quota of microcystin. This technique could be an effective approach for the routine monitoring of the most at-risk water bodies.

Toxic cyanobacterial blooms, as well as their increasing global occurrence, pose a serious threat to human health, domestic animals, and livestock. The frequency and severity of bloom events continue to rise, most probably as a direct result of increased nutrient loading of water systems worldwide. The number of lakes in Quebec, Canada, affected by blooms of 2 × 10^4 cells ml^{-1} or more has been increasing from 21 (2004) to 28 (2005), 62 (2006), 157 (2007), 138 (2008), and 150 (2009). Government agencies are under tremendous pressure to cope with escalating demands for water analysis, specifically, for cyanotoxins.

In Missisquoi Bay, Lake Champlain, public health advisories have been issued from 2001 to 2009 resulting in the closure of several beaches and periodic no-drinking warnings for the water. These advisories forbid any direct contact with the lake water by both people and animals because of the presence of cyanotoxins. The economic impact around the lake has been substantial, with revenues sometimes falling by 40 to 80% (20).

For the past 8 years, microcystin (MCYST)-producing cyanobacterial genera have composed a major part of the bacterial community in Missisquoi Bay during both the summer and the fall. To date, five species known to produce toxins based on the literature (2) have been identified in the lake, including species of *Microcystis* and *Anabaena* (27).

All species with microcystin-producing strains also include related strains that lack the ability to produce this toxin. The nonproducing strains cannot be differentiated by traditional microscopy or ribosomal gene sequences (15). Reliable tools to detect and characterize toxin-producing cyanobacteria are required. Enzyme-linked immunosorbent assay (ELISA) and high-pressure liquid chromatography (HPLC) are currently the most widely used techniques to evaluate whether toxins are present in water samples. The risk assessment response to the increasing occurrence of cyanotoxins has been seriously constrained due to the limited number of available standards and the limited analytical capability of some laboratories. At least 89 microcystin analogues have been characterized (35), but fewer than 10 reference standards are currently available.

The development and validation of increasingly sensitive, specific, and reliable molecular tools will contribute to the next generation of monitoring approaches. The detection and quantitation of specific target genes, such as those involved in the synthesis of toxins in cyanobacteria, are the cornerstone of new techniques to identify, monitor, or profile specific targets in environmental samples. These approaches are in general less expensive and faster than the currently available chemical assays and do not rely on reference standards.

The structure of the microcystin biosynthesis cluster of two

* Corresponding author. Mailing address: National Research Council Canada, Biotechnology Research Institute, 6100 Royalmount Ave., Montreal, QC H4P 2R2, Canada. Phone: (514) 496-6182. Fax: (514) 496-6265. E-mail: charles.greer@cnrc-nrc.gc.ca.

▽ Published ahead of print on 18 June 2010.

strains of *Microcystis aeruginosa* (17, 18, 31), *Planktothrix agardhii* NIVA CYA 126/8 (3), and *Anabaena* sp. strain 90 (28) which encodes the nonribosomal peptide synthetase-polyketide synthase enzyme complex has been elucidated. The *mcy* gene cluster is located on the chromosome and contains 10 genes (*mcyA* to *mcyJ*). The two polyketide synthase modules of *mcyG* and *mcyE*, together with the two polyketide synthase modules of *mcyD*, are responsible for the synthesis of the unique Adda moiety of microcystins. The Adda side chain is largely responsible for the toxicity through protein phosphatase inhibition (7, 8, 12).

The number of water bodies affected by cyanobacterial blooms has been increasing worldwide, and scientists have designed primers for the various genes involved in the biosynthesis of microcystins. The *mcyD* gene has been used in conventional PCR for phylogenetic studies (16, 22, 29) and as a target to characterize cyanobacterial blooms in Lakes Ontario (11) and Erie (19). The *mcyE* gene has also been targeted to design genus-specific primers (23, 24, 33) and universal primers encoding the aminotransferase domain of various genera of cyanobacteria (4, 13). All of these primers generated PCR fragments that were larger than the recommended size for quantitative PCR (Q-PCR) (100 to 200 bp).

A Q-PCR technique, the *Taq* nuclease assay, was developed by Rinta-Kanto and colleagues (25, 26) to study the distribution and abundance of toxic *Microcystis* blooms in western Lake Erie. In their first study, the *mcyD* probe was highly specific to *Microcystis* species but failed to detect the *mcyD* gene in one of the samples that had a detectable concentration of microcystin in the water. Other microcystin-producers, such as *Anabaena* and *Planktothrix*, were identified in that sample and were likely responsible for toxin production.

The number of gene sequences related to microcystin biosynthesis in the databases has been increasing rapidly since the beginning of 2000. The objectives of this study were to develop a rapid, Q-PCR-based technique for detecting and monitoring the dynamics of microcystin-producing cyanobacteria and to determine the correlation between toxigenic cells and toxin concentration. The *mcyD* gene was selected as the specific target for characterizing cyanobacterial blooms and applied in Missisquoi Bay, Lake Champlain, during the summers of 2006 and 2007. Oligonucleotide primers were designed based on the alignments of all 50 *mcyD* nucleotide sequences available in GenBank as of March 2006 and were verified in December 2009. The alignments revealed that the polyketide synthase sequences were divided into three major clusters: some of the submitted sequences encoded the first dehydratase domain, others were from one of the β-ketoacyl synthases and the third group of sequences encoded part of both the ketoacetyl synthase and the acetyltransferase domains. Primers were designed to create PCR fragments from two different regions of the *mcyD* gene. These fragments were cloned to create a standard curve for absolute quantification. This strategy was chosen to ensure that the quantification of cells carrying the target gene would be performed with a standard curve that originated from a single gene copy.

MATERIALS AND METHODS

Sampling. Integrated water samples were collected in 2006 and 2007 in Missisquoi Bay, Lake Champlain, from two different locations in Philipsburg representing littoral (45°02.380′N, 73°04.773′W) and pelagic (45°02.363′N, 73°05.802′W) sites over the whole growing season between mid-May and the end of October (biweekly). Integrated water samples were collected with a peristaltic pump from all depths of the euphotic zone (this lake is unstratified) in a clean polypropylene bottle. Water samples for pigment and toxin analysis were collected on 47-mm Whatman 934-AH filters (Whatman, Inc., Florham Park, NJ). Phytoplankton subsamples (100 ml) for enumeration were preserved with 0.5 ml of Lugol's iodine. Counts were performed using an inverted microscope by the Utermohl method (32).

Extraction for toxin analyses. Water samples (1 liter) were stored at 4°C until filtered on 1.2-μm grade GF/C glass microfiber membranes within 5 h of sampling. The membranes were kept frozen at −20°C until processed. Filters were extracted by accelerated solvent extraction (ASE) according to the procedure described by Aranda-Rodriguez et al. (1). Briefly, each filter containing the cyanobacteria was placed in an 11-ml stainless steel ASE cell which was then filled with hydromatrix beads (Varian, Palo Alto, CA). Extraction took place in an ASE 200 (Dionex Co., Oakville, Canada) with 2 cycles of 5 min of static extraction at 2,000 lb/in^2 and 80°C using 75% methanol. Extracts (27 ml) were spiked with 50 μl formic acid and evaporated to dryness using a Zymark Turbovap LV concentrator (Caliper Life Science, Hopkinton, MA) and reconstituted with 1 ml of 50% methanol for further analysis.

HPLC-PDA analysis. The chromatographic separation of extracts was performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with a Zorbax SB-C_{18} column (250 mm by 4.6 mm, 5-μm particle size). The mobile phase and eluting gradient program followed that described by Aranda-Rodriguez et al. (1). Detection was achieved with a PD40 photodiode array (PDA) detector (Dionex), UV spectra were acquired from 200 to 300 nm, and data were recorded at 238 nm. Positive microcystin analogue peak confirmation was obtained when the retention times matched those of the standards (RR, YR, LR, LA, LW, and LF) and had the characteristic UV spectra.

ELISA. Based on the HPLC-PDA results, samples were diluted with deionized water to obtain a final concentration within the calibration range of the kit. Tests were performed using an Envirologix QuantiPlate test kit for microcystins (Envirologix, Portland, ME), following the manufacturer's instructions. Absorbance was measured at 450 nm/630 nm using a Multiskan spectrum (Thermo Electron Co., Vantaa, Finland). The detection limit of the ELISA kit was 0.175 μg liter^{-1}. Large volumes of water were filtered (up to 1 liter) to ensure that the samples would be within the detection limit. Based on a 1-liter volume, samples with microcystin concentrations equal to or above 0.175 pg ml^{-1} would fall within the detection limit.

Liquid chromatography-tandem mass spectrometry (LC–MS-MS) analysis. The chromatographic separation of extracts was performed using a Finnigan Surveyor Plus HPLC system (Thermo Electron Corporation, San Jose, CA) equipped with a Zorbax 300SB-C_{18} column (150 mm by 2.1 mm, 5-μm particle size). The mobile phase consisted of 0.1% formic acid in 5% aqueous acetonitrile (mobile phase A) and 0.1% formic acid in acetonitrile (phase B). Solvents were degassed online, and the column temperature was maintained at 30°C. The elution gradient started with 100% phase A for 2 min, followed by an 8-min linear gradient to 100% B, then a 2-min hold at 100% phase B, and returned back to 100% phase A in 3 min, at a flow rate of 200 μl min^{-1}. The system was equilibrated for 5 min at the initial conditions before the next injection. The sample injection volume was 10 μl.

Mass spectrometric experiments were performed using a Thermo Finnigan TSQ Quantum Ultra EMR triple quadrupole mass spectrometer (Thermo Electron Corporation). The samples were analyzed in positive-ion electrospray ionization, using the selective reaction monitoring (SRM) mode. Xcalibur version 2 was used for data acquisition and processing. The following SRM transitions (*m*/*z*) for microcystin were monitored: MCYST→LR (995→135), MCYST→LA (910→776), MCYST→RR (520→135), MCYST→LF (986→852), MCYST→LW (1025→446), and MCYST→YR (1045→135).

DNA extraction. To produce the Q-PCR standard curves, DNA was extracted from pure cultures of *Microcystis aeruginosa* UTCC 299 and UTCC 300 (University of Toronto, Culture Collection) using the method of Hisbergues et al. (10) with the following modifications: 50 ml of culture was filtered on a 0.2-μm modified polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA). The membrane was transferred to 5 ml of lysis buffer containing 5 mg ml^{-1} of lysozyme and incubated for 1 h in a 37°C incubator with gentle mixing on a Roto-Torque rotator (Cole-Parmer, Anjou, Canada). After the proteinase K-SDS treatment, the membrane was removed from the tube and the solution was extracted with one volume of chloroform/isoamyl alcohol (24:1). RNase A was added to the supernatant at a final concentration of 100 ng μl^{-1}. The solution was incubated at 37°C for 1 h and then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). DNA

was precipitated overnight at −20°C in 1 volume of cold 2-propanol and then centrifuged at 12,100 × *g* for 30 min. The pellet was washed with 70% (vol/vol) ethanol, air dried, and resuspended in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8).

Water samples (100 ml) were stored at 4°C until filtered on 0.2-μm hydrophilic polyethersulfone membranes (PAL Corporation, Ann Arbor, MI) within 24 h of sampling. The membranes were kept frozen at −20°C until processed. DNA extraction was performed by transferring the membrane to 5 ml of lysis buffer (50 mM Tris-Cl, 50 mM EDTA, pH 8, 50 mM sucrose, 0.5% SDS, 100 mM NaCl) containing 5 mg ml^{-1} of lysozyme. Samples were incubated for 1 h in a 37°C incubator with gentle mixing on a Roto-Torque rotator (Cole-Parmer). After the addition of 0.5% SDS and 0.4 mg ml^{-1} proteinase K, the samples were incubated on the rotator (37°C) for another 2 h. The membrane was dissolved with one volume of prewarmed phenol/chloroform/isoamyl alcohol (25:24:1) following an incubation of 10 min at 56°C. The samples were centrifuged for 5 min at 3,823 × *g*. One volume of chloroform/isoamyl alcohol (24:1) was added to the supernatant. The mixture was incubated for 10 min at 56°C and then centrifuged as before. RNase A was added to the supernatant at a final concentration of 100 ng μl^{-1}. The samples were incubated for 1 h at 37°C and then subjected to one phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by one chloroform/isoamyl alcohol (24:1) extraction. The DNA was precipitated overnight with 1 volume of cold 2-propanol and then centrifuged at 12,100 × *g* for 30 min. The pellet was washed with 70% (vol/vol) ethanol, air dried, and resuspended in TE (pH 8).

PCR amplification and cloning. Primers were designed for the β-ketoacyl synthase and the first dehydratase subunits of the *mcyD* gene (*mcyD*$_{KS}$ and *mcyD*$_{DH}$, respectively), which are essential for the biosynthesis of microcystin (7, 8, 12). ClustalW multiple alignment analyses (30) were performed with all of the *mcyD* sequences available in GenBank as of March 2006, to identify the highly conserved regions of the various subunits. The primers designed for the β-ketoacyl synthase were *mcyD*$_{KS}$F1, 5′-TGG-GGA-TGG-ACT-CTC-TCA-CTT-C-3′, and *mcyD*$_{KS}$R1, 5′-GGC-TTC-AAC-ATT-CGG-AAA-ACG-3′, which generated a fragment of 107 bp. The primer sequences for the first dehydratase subunit were *mcyD*$_{DH}$F1, 5′-TAC-GGG-AGT-AAC-TTT-CGG-CTC-A-3′, and *mcyD*$_{DH}$R1, 5′-ACA-AGC-ATC-TAA-CAT-AGC-GGG-A-3′, which generated a PCR fragment of 129 bp. The *mcyD*$_{KS}$ and *mcyD*$_{DH}$ primers were designed to be perfect matches with *Microcystis aeruginosa* strain UTCC 299 and *Microcystis aeruginosa* UTCC 300, respectively.

The PCR mixture (50 μl) contained 25 ng of genomic DNA from *Microcystis aeruginosa* UTCC 299 or UTCC 300, 50 pmol of each oligonucleotide primer, 2.5 units of *Taq* DNA polymerase (GE Healthcare, Baie d'Urfe, Canada), 5 μl of 10× *Taq* polymerase buffer (100 mM Tris-Cl, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$), 1 mM $MgCl_2$, 8 μl of 1.25 mM deoxynucleoside triphosphates (200 μM [each] dATP, dGTP, dCTP, and dTTP), and 0.13 μg μl^{-1} bovine serum albumin (BSA). Prior to the addition of *Taq* DNA polymerase, the samples were denatured for 5 min at 96°C. For PCR cycling, denaturation was carried out at 94°C for 1 min, the annealing time was 1 min at 58°C, and primer extension was carried out at 72°C for 1 min. Thirty cycles were performed with a final extension at 72°C for 10 min. The PCR products were purified with a Montage PCR kit (Millipore) and quantified by SYBRSafe staining (Qiagen, Mississauga, Canada) and spot densitometry using a ChemiImager (Alpha Innotech Corporation, San Leandro, CA).

The amplification products were ligated into the pDrive cloning vector (Qiagen) and transformed with XL1 Blue competent cells following the method described by the manufacturer (Stratagene, La Jolla, CA). Transformants were screened on LB plates containing 100 μg ml^{-1} ampicillin, 20 μg ml^{-1} 5-bromo-4-chloro-3 indolyl-β-D-galactoside (X-Gal), and 0.25 mM isopropyl-β-D-thiogalactoside (IPTG). Recombinant plasmid DNA was extracted using a QIAprep spin miniprep kit (Qiagen).

Standard curve for *mcyD* quantification and Q-PCR analyses. Standard curves showing the relationship between the *mcyD* copy numbers and threshold cycles (C_T) were generated with serial dilutions of plasmid DNA containing part of the *mcyD*$_{KS}$ or *mcyD*$_{DH}$ gene. Prior to quantification, the plasmids were linearized with the restriction endonuclease SmaI. The plasmid DNA concentrations were evaluated by fluorescence using PicoGreen (Invitrogen, Burlington, ON, Canada) and a Safire microplate detection system (Tecan, Männedorf, Switzerland). The concentrations were also validated on an agarose gel by SYBRSafe staining and spot densitometry with a serial dilution of the 1-kb DNA ladder from MBI Fermentas (Amherst, NY).

Tenfold serial dilutions of plasmid DNA in triplicate were used to establish the mean C_T values. The plasmid DNA was diluted in 5 mM Tris-Cl, pH 8.0. The standard curves were prepared with dilutions ranging from 2.31×10^1 to 2.31×10^6 copies of the *mcyD*$_{KS}$ gene μl^{-1} and from 2.29×10^1 to 2.29×10^6 copies of the *mcyD*$_{DH}$ gene μl^{-1}. To evaluate the *mcyD* gene copy number of the plasmid DNA, the number of picomoles of plasmid per ng of DNA was calculated using the following formula: 1×10^6 pg $\mu g^{-1} \times 1$ pmole 660 $pg^{-1} \times 1$ plasmid $size^{-1}$. The DNA gene copy per ng of total DNA was then determined as follows: pmole plasmid ng^{-1} DNA × 6.022×10^{23} molecules mol^{-1} (Avogadro constant) × 1×10^{-12} moles $picomole^{-1}$. Amplification and detection were performed using a Rotor Gene 3000 instrument with software version 6.0 (Corbett Research, Mortlake, NSW, Australia) in a 20-μl volume containing 4 mM $MgCl_2$, 0.8 μM of both primers, 5 μl of SYBR green from a QuantiTect PCR kit (Qiagen), and 5 μl of template DNA. The amplification conditions were as follows: preheating at 95°C for 10 min and then 45 cycles of 58°C for 15 s and 72°C for 20 s. Fluorescence was measured at the end of each cycle at 72°C. The PCR efficiency and the correlation coefficient of both the *mcyD*$_{KS}$ and the *mcyD*$_{DH}$ standard curve were 0.95 and 0.999, respectively.

The limit of detection (LOD) was determined for the Q-PCR assay used in this study. Briefly, Q-PCRs of a 2-fold dilution series of linearized plasmid containing the gene fragment of interest were performed in 6 replicates, starting with a concentration of approximately 1,000 gene copies/reaction mixture volume to a concentration of less than 1 gene copy/reaction mixture volume. The results were used to determine the number of copies that is necessary for reliable detection of the gene using the LOD tool from GenEx Pro software (version 4.4.2; MultiD Analyses AB, Göteborg, Sweden). When a cutoff value of 33 cycles and a level of confidence of 95% were applied, the LOD was 2.23 copies μl^{-1} of reaction mixture.

The *mcyD* gene copy numbers of Missisquoi Bay water samples were determined by Q-PCR immediately after DNA quantification by PicoGreen. The DNA concentration was also verified on an agarose gel by SYBRSafe staining and spot densitometry with a serial dilution of lambda DNA (GE Healthcare). The DNA was diluted to 5 to 10 ng μl^{-1} (10^0) with 5 mM Tris-Cl, pH 8.0, prior to preparing the 10-fold serial dilutions. The water samples were analyzed in duplicate as 10^0 to 10^{-2} or 10^{-1} to 10^{-3} dilution series. In each run, triplicates of the 10^{-5} and/or 10^{-6} plasmid DNA and 5 mM Tris-Cl, pH 8.0, were used as positive and negative controls, respectively. The water sample C_T values were determined by importing the standard curve and adjusting it to the C_T values of the 10^{-5} or 10^{-6} plasmid DNA standard used as the positive control.

Statistical analysis. Pearson correlation coefficients (r) between the *mcyD*$_{KS}$ or *mcyD*$_{DH}$ gene copy number and the microcystin concentration were calculated with the statistical software R, version 2 (R Project for Statistical Computing). Predictive regression models relating the *mcyD*$_{KS}$ gene copy number to the microcystin concentration and the microscope-based cell counts were developed using SAS JMP 8. Data were transformed to logarithms when necessary to meet the assumptions of regression and to derive exponential growth rates.

RESULTS

Q-PCR analysis with *mcyD*$_{KS}$ in Missisquoi Bay in 2006 and 2007. Copies of *mcyD*$_{KS}$ were detected by Q-PCR on every sampling date at both stations during the summer of 2006: in the littoral station, more than 6.5×10^4 copies of the *mcyD*$_{KS}$ gene ml^{-1} were detected in August, and an average of 4.0×10^4 copies ml^{-1} were detected in September; the microcystin concentrations were more than 4 ng ml^{-1} and approximately 2 ng ml^{-1}, respectively (Fig. 1A). Although water samples from the pelagic zone could not be collected in mid-August because of strong winds, two bloom events ($>2 \times 10^4$ cells ml^{-1}) were also identified in that station but with a different trend: the toxin concentrations were significantly lower than the *mcyD*$_{KS}$ gene copy number (Fig. 1B). Prior to the first bloom event, 3.0×10^4 copies of the *mcyD*$_{KS}$ gene ml^{-1} were detected when the microcystin concentration was only 0.5 ng ml^{-1}. There was a good correlation at both stations between gene copy numbers and microcystin concentrations (for ELISA results, $r = 0.93$, $P < 0.001$ [littoral] and $r = 0.87$, $P < 0.001$ [pelagic]; for HPLC results, $r = 0.89$, $P < 0.001$ [littoral and pelagic]).

The amount of toxin per gene copy increased steadily with time (Fig. 2A). There was a highly significant correlation between gene copy number and microcystin concentration from the end of May until mid-October (for ELISA results, $r = 0.93$, $P < 0.001$ [littoral] and $r = 0.91$, $P < 0.001$ [pelagic]). A closer

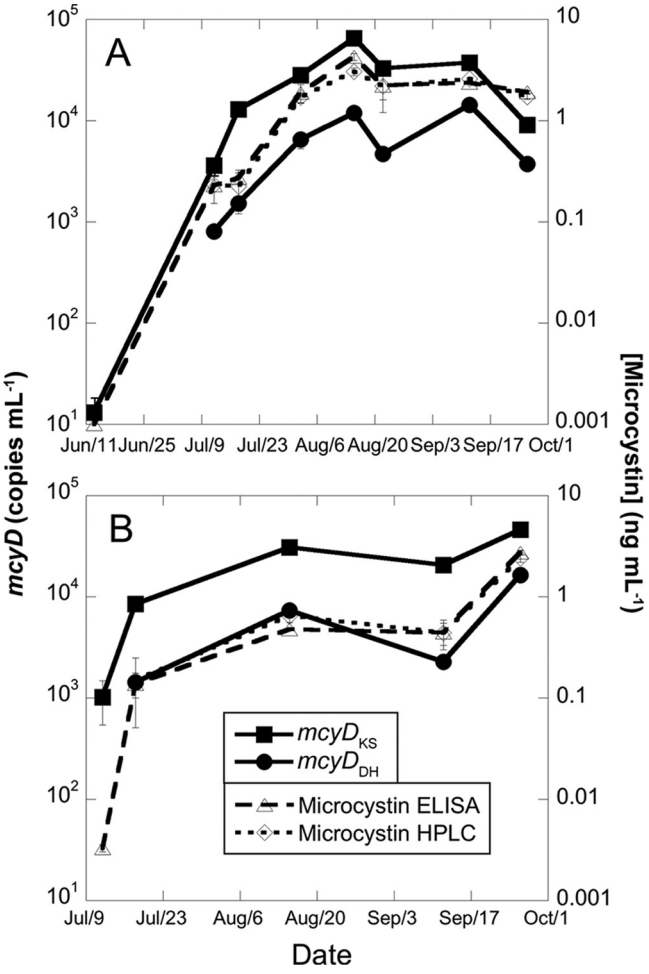

FIG. 1. Results of quantitative PCR analysis of the $mcyD_{KS}$ gene in relation to the results of standard chemical methods (ELISA and HPLC) used to detect microcystin in the littoral (A) and pelagic (B) zones of Philipsburg, Missisquoi Bay, during the summer of 2006.

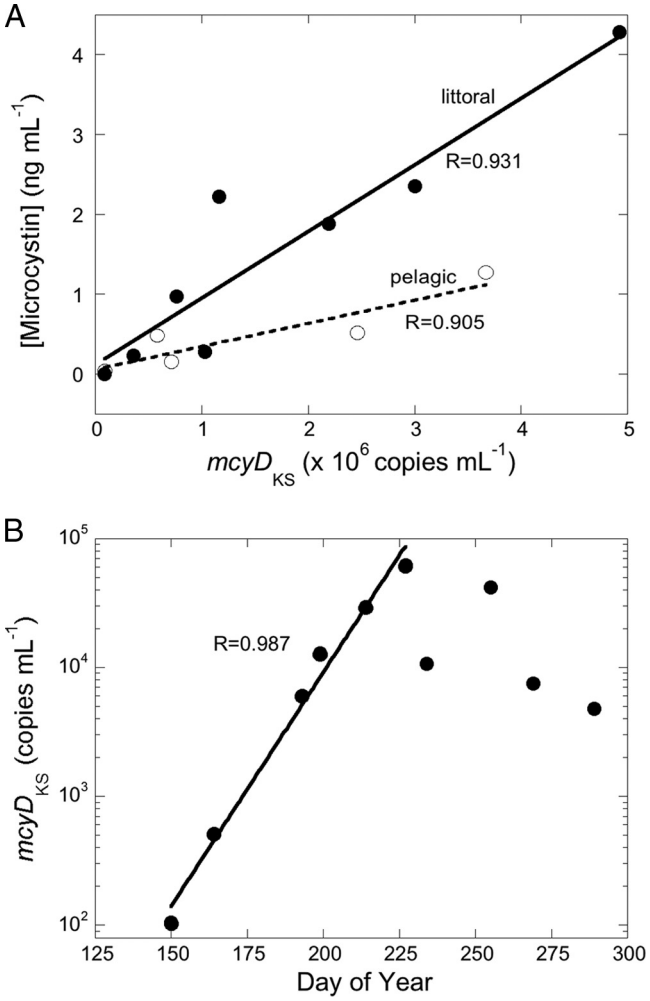

FIG. 2. (A) Microcystin concentrations measured by ELISA versus $mcyD_{KS}$ gene copy numbers by Q-PCR in Missisquoi Bay during the 2006 growing season, from May 30 to October 16, in the littoral and pelagic stations. (B) There was an extended period of exponential increase in the $mcyD_{KS}$ gene copy number in Missisquoi Bay from May 30 (day 150) to August 15 (day 227), shown by the straight regression line on this semilog plot. Correlation coefficients are indicated in the panels.

look at the Q-PCR results showed that there was an extended, nearly perfect exponential increase in gene copy numbers from late spring to mid-summer 2006 (Fig. 2B). The exponential growth corresponded to an increase of a factor of exp(0.072) $mcyD_{KS}$ copies per day, or approximately 7.5% per day, from May 30 (day 150) to August 15 (day 227), when the maximum was reached, a period of uninterrupted growth of 77 days.

In contrast to 2006, in 2007, cyanobacterial blooms were not observed until the month of September. A level 1 public health advisory was issued only on September 7 (2×10^4 cells ml^{-1}). The toxin concentrations were low throughout the summer, reaching an average of 7.2×10^{-3} ng ml^{-1} on August 2 in the littoral station (Fig. 3), where a similar trend was observed between the $mcyD_{KS}$ gene copy number and the toxin concentration. At the beginning of August, Q-PCR detected approximately 3×10^2 copies of the $mcyD_{KS}$ gene when the microcystin concentration was 7.2×10^{-3} ng ml^{-1}. In the pelagic zone, the number of $mcyD_{KS}$ gene copies was higher than the toxin concentration at the beginning of the summer but only until the end of August. A peak of approximately 2.5×10^2 $mcyD_{KS}$ gene copies ml^{-1} was observed on August 30, but the corresponding toxin concentration was quite low. The toxin concentrations increased slightly afterwards, whereas the number of $mcyD_{KS}$ gene copies continued to decrease. There was no significant correlation between gene copy number and microcystin concentration.

Q-PCR analysis with $mcyD_{DH}$ in Missisquoi Bay in 2006. The trends described for the $mcyD_{KS}$ target were also observed in both stations with the $mcyD_{DH}$ gene (Fig. 1A and B). The major difference was that the $mcyD_{DH}$ gene copy numbers were much lower than the $mcyD_{KS}$ numbers. For example, slightly more than 10^4 copies of the $mcyD_{DH}$ gene were detected in the littoral zone during the first bloom event, compared to 6.5×10^4 copies of the $mcyD_{KS}$ gene (Fig. 1A). There was a good correlation at both stations between gene copy number and microcystin concentration (for ELISA results, $r = 0.74$, $P < 0.05$ [littoral] and $r = 0.95$, $P < 0.05$ [pelagic]; for

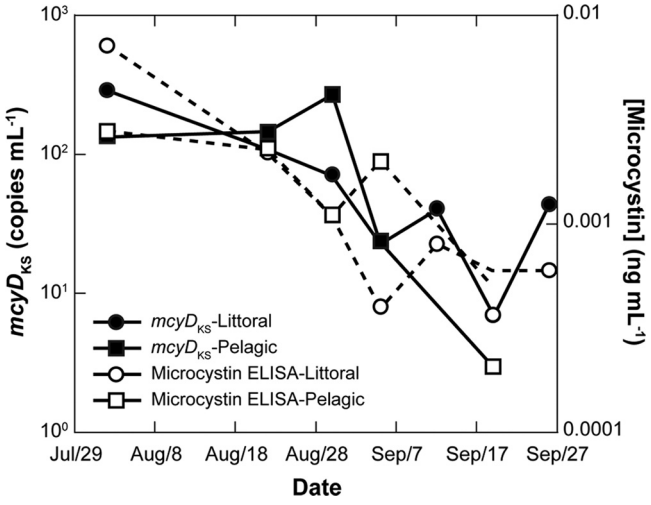

FIG. 3. Results of quantitative PCR analysis of the $mcyD_{KS}$ gene in relation to the results of the chemical method ELISA used to detect microcystin in the littoral and pelagic zones of Philipsburg, Missisquoi Bay, during the summer of 2007.

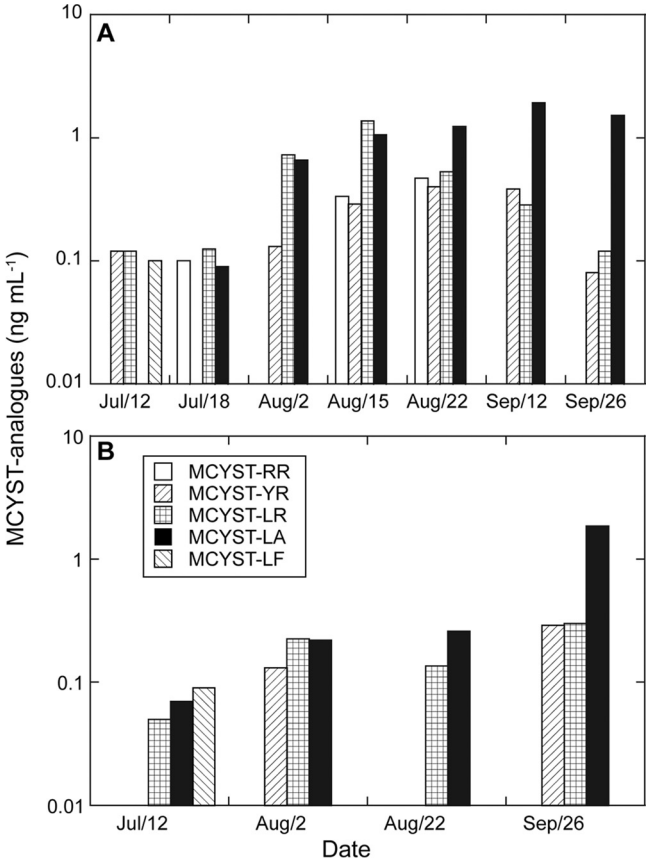

FIG. 4. Results of HPLC analysis of MCYST analogues identified in Philipsburg, Missisquoi Bay, during the summer of 2006. (A) Littoral station. (B) Pelagic station.

HPLC results, $r = 0.88$, $P < 0.005$ [littoral] and $r = 0.97$, $P < 0.005$ [pelagic]) throughout the summer.

Microcystin analyses. In 2006, the ELISA and HPLC chemical assays yielded similar results, with the exception of the littoral station during the August bloom, where the toxin concentration obtained by HPLC was lower than the results with ELISA (Fig. 1A). Four microcystin analogues (RR, YR, LR, and LA) were identified by HPLC during the August bloom, MCYST LR and LA being predominant on August 15 at the littoral station (Fig. 4A and B). A steady increase of the MCYST LA analogue was observed throughout the summer. This analogue dominated the bloom in September, reaching a concentration of almost 2 ng ml^{-1}. The HPLC results were validated by the results of LC-MS analyses (data not shown).

The ELISA results for 2006 demonstrated that the toxin concentration increased by 15% per day for 2 months ($r = 0.999$). This allowed us to predict in early July that the lake would close for drinking water on July 26 if the trend continued. The actual health advisory was issued on July 27.

Cyanobacterial abundance in 2006. Several cyanobacterial genera, including potential microcystin-producing species such as *Microcystis aeruginosa*, *Microcystis wesenbergii*, and *Anabaena spiroides*, were identified by microscopy (Fig. 5 and 6). *Microcystis aeruginosa* cells were first identified from both stations at the end of May and became numerically dominant throughout the summer and early fall. The population followed the same trend as the number of $mcyD_{KS}$ copies and microcystin concentration in the littoral zone (Fig. 1A and 5A). The highest number of cells was recorded on August 15, when the relative abundance reached more than 1.57×10^5 cells ml^{-1} (Fig. 5A). On that date, the *M. aeruginosa* population accounted for 58% of the total cyanobacterial abundance. The population dropped to 7.02×10^4 cells ml^{-1} on August 22 but peaked again at the beginning of September, with more than 1.37×10^5 cells ml^{-1} on September 12. On those dates, the population corresponded to 46% and 69% of the total cyanobacterial abundance, respectively. Six different species of *Anabaena* were identified throughout the summer (5, 21). On August 2, prior to the first bloom, the *Anabaena spiroides* population accounted for 36% and 40% of the total cyanobacterial abundance in the littoral and pelagic stations, respectively (Fig. 5A and B). During the bloom of August 15, the *A. spiroides* population constituted 21% of the total abundance in the littoral station, at 5.61×10^4 cells ml^{-1}, but during the second bloom event, the *A. spiroides* population made up only 4% of the total abundance in both stations. *Aphanizomenon flos aquae* was first detected in July and remained part of the phytoplankton community until late fall. The highest counts were obtained in the littoral station at the beginning of October, when *A. flos aquae* cells constituted 58% of the total cyanobacterial abundance (data not shown).

Cyanobacterial abundance in 2007. The cyanobacterial abundance was substantially lower in 2007 than in 2006. *Microcystis aeruginosa* cells were first identified on June 6 in the littoral zone and on July 19 in the pelagic zone (data not shown). The highest numbers of cells were recorded on August 22, with more than 3.8×10^3 and 1.5×10^3 cells ml^{-1} in the littoral and pelagic stations, respectively (Fig. 6A and B). On this date, the *M. aeruginosa* population accounted for 4.9% (littoral) and 1% (pelagic) of the total cyanobacterial abundance. *M. aeruginosa* cells were not detected after September 5. Six species of *Anabaena* were identified in both stations

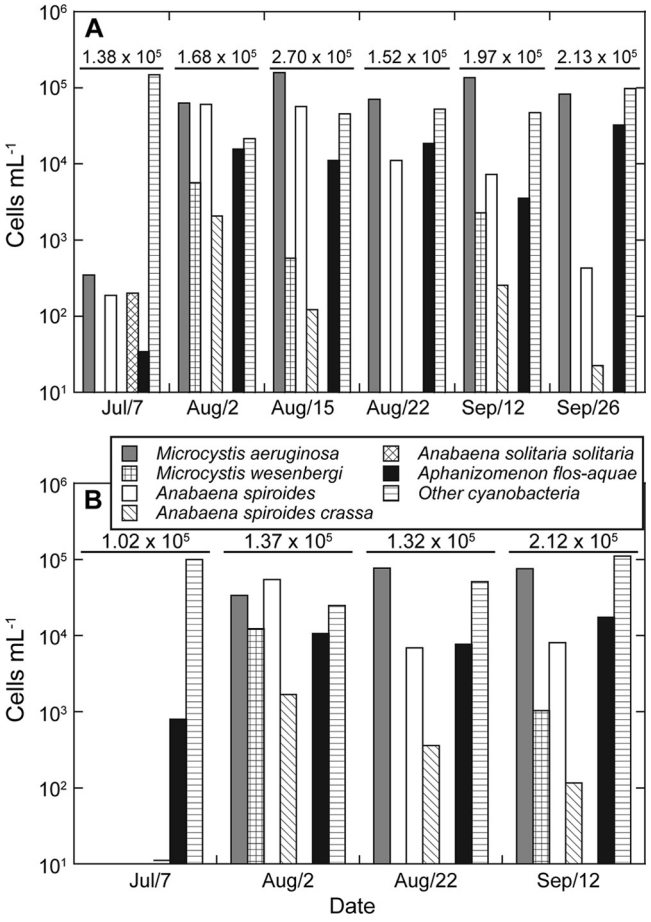

FIG. 5. Abundance of potential toxin-producing cyanobacteria in Missisquoi Bay during the summer of 2006. (A) Littoral station. (B) Pelagic station. The numbers above the bars represent the total cyanobacterial abundance (cells ml^{-1}).

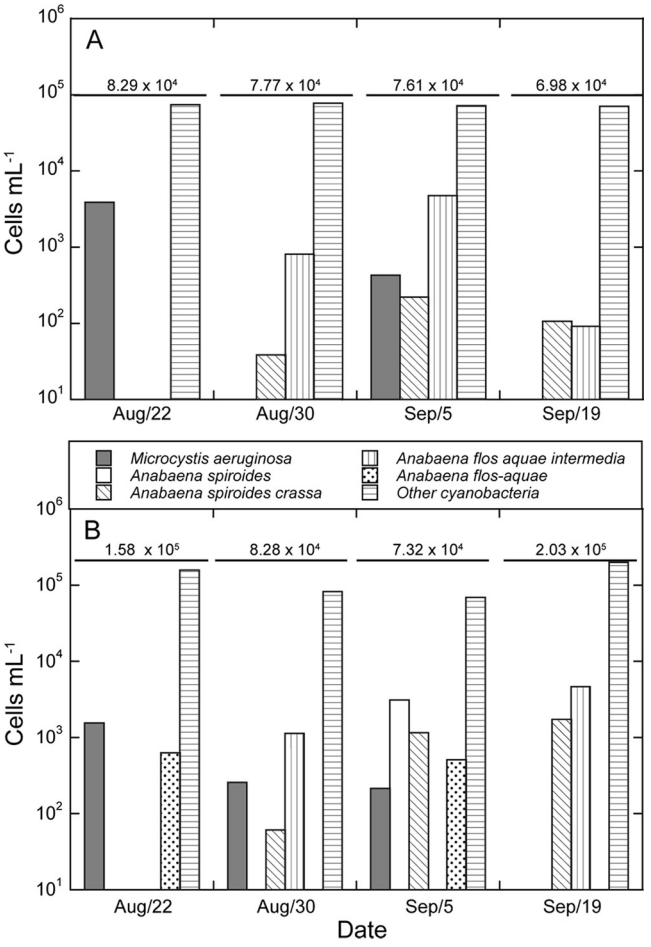

FIG. 6. Abundance of potential toxin-producing cyanobacteria in Missisquoi Bay during the summer of 2007. (A) Littoral station. (B) Pelagic station. The numbers above the bars represent the total cyanobacterial abundance (cells ml^{-1}).

during the summer of 2007. *Anabaena flos aquae intermedia* was identified in both stations but seemed to be predominant at the beginning of September in the littoral zone, at 4.69×10^3 cells ml^{-1}. *Anabaena spiroides crassa* was first detected at the end of August, and the number of cells increased until mid-September, from 6.01×10^1 to 1.71×10^3 cells ml^{-1}, in the pelagic station (Fig. 6B). Three different species of *Aphanizomenon* were identified at the beginning of the summer, but none were detected after August 2.

DISCUSSION

Oligonucleotide primers for the $mcyD_{KS}$ gene, encoding β-ketoacyl synthase, were designed and used successfully to characterize cyanobacterial blooms in Missisquoi Bay, Lake Champlain. A higher number of *mcyD* copies was generated with these primers than with the primers designed from the first dehydratase subunit, $mcyD_{DH}$. Similar results were obtained from Constance Lake, ON, a much smaller, shallow mesotrophic lake sampled during the same time frame (data not shown). This difference in detection could be attributed to nucleotide mismatches in the reverse primer of $mcyD_{DH}$. Alignments of the $mcyD_{DH}$ sequences revealed 2 mismatches between the primer sequence of the reference strain and the sequence of the *Microcystis aeruginosa* that was identified in the lake. These results show that primer design is a critical step in the development of Q-PCR methodology for the analysis of cyanotoxins.

The Q-PCR method developed in this study allowed the detection of microcystin-producing cyanobacteria as early as the end of May during the summer of 2006. At the littoral station, toxin producers were detected by Q-PCR before this was possible by chemical analyses (ELISA and HPLC) or microscopy. Furthermore, there was a highly significant correlation between gene copy numbers and microcystin concentrations. The precision afforded by toxin gene tracing permitted us to document a quite extraordinary period of exponential increase in microcystin-producing cells that was maintained over 50 days. This uninterrupted run was followed by another period of slightly reduced growth, at 6% per day, lasting for another 27 days. When the maximum was reached, *Microcystis aeruginosa* made up 46% of phytoplankton biomass, and *Anabaena spiroides* was at 45%. This smooth uninterrupted growth phase suggests that external influences (changes in weather, for example) were minimal once the conditions were

appropriate for cell growth. The bloom was arrested only when essentially all phytoplankton biomass was made up by large, colonial, buoyant cyanobacteria.

The proportions of *M. aeruginosa* in both the littoral and the pelagic stations were very similar during the months of August and September. There were tremendous spatial variations in total cell abundance, however, so that toxin concentrations varied in August and September from 0.5 ng ml^{-1} in the pelagic station to more than 2 ng ml^{-1} in the littoral station. Southwesterly winds are predominant in this area and often contribute to the accumulation of scum near the shore at our littoral station. The littoral zone also has shallow and warm water, which provides highly favorable conditions for the growth of cyanobacteria. The regression analysis of the microcystin concentrations by ELISA versus $mcyD_{KS}$ gene copy numbers did indicate that nearshore accumulations of cyanobacteria contained more toxin per cell, estimated at 870 fg per gene copy, than did open water populations, which averaged 300 fg per gene copy. These are not unusual values, but they are higher than most microcystin cell quotas reported in the literature, which hover around 200 fg per cell (6). This spatial segregation suggests physical selection of different phenotypes of toxigenic cells, if not genotypes, by wind. If so, it would provide interesting confirmation of the correlation between colony size, buoyancy, and cell-specific toxicity that has been demonstrated for *Microcystis* in a German lake (14).

The strength of the relationship between the numbers of $mcyD_{KS}$ gene copies detected in May and June and toxin concentrations in the lake could be used to predict the date of a public health advisory based on the Canadian drinking water guideline of 1.5 μg of microcystin $liter^{-1}$. The gene copy number equation suggested that the health advisory would be issued on July 21, 1 week prior to the actual closing date of July 27. The ELISA results demonstrated that the toxin content increased at a fairly constant 15% per day over the same 2 months. A regression equation of the microcystin concentration measured by ELISA versus the day of the year suggested that a public health advisory would be posted on July 26. The accuracy of these predictions, based on early season dynamics, would not be expected every year but is sufficiently striking to encourage further work on toxic bloom prediction via these sensitive methods.

The strong linear relationship between toxin concentrations and gene copies in the two zones does not tell the whole story of toxin dynamics, however. Another interesting observation was that the amount of toxin per gene copy increased steadily with time. The toxin concentration increased faster (+15% per day) than the gene copy number (+7.5% per day) or the *Microcystis* population (+7.5% per day). This might occur if (i) there is a succession of strains of increasing toxicity with time, (ii) the toxigenic cells slowly accumulate and sequester toxin with time, or (iii) gene expression is enhanced with time. We are unable to distinguish among these possibilities on the basis of these data. Nevertheless, the fraction of *Microcystis* cells that were potentially toxic (i.e., contained the gene) was more or less constant (except for some unusual outliers where few cells seemed toxic). This suggests that *Microcystis* was the principal genus producing microcystin in Missisquoi Bay during our study.

The HPLC profile revealed a change in the toxin composition during the two bloom events. Four microcystin analogues were observed during the bloom in August. Changes in the toxin composition have also been observed by Watanabe et al. (34) from a batch culture experiment containing two *Microcystis* species. Similar amounts of MCYST RR, YR, and LR were present at the beginning of the exponential growth phase of *M. viridis*, but a significant increase from 35 μg to 112 μg of MCYST RR per 100 mg of cells was recorded during the late stage of its exponential growth. In a similar experiment, a substantial increase of MCYST YR from 130 μg to 1,020 μg per 100 mg of cells was observed during the exponential growth phase of a highly toxic strain of *M. aeruginosa*.

Favorable conditions for a cyanobacterial bloom occurred only late in the summer of 2007. Very low concentrations of microcystin were monitored with the ELISA method during the month of September. *M. aeruginosa* was identified early in the summer in both the pelagic and littoral stations but never reached more than 4×10^3 cells ml^{-1} throughout the summer. Contrary to the situation in the fall of 2006, the cyanobacterial abundance was dominated by various species of *Anabaena* in September 2007.

In 2007, the low correlation between gene copy number and microcystin concentration in the littoral zone and the lack of correlation in the pelagic zone could be attributed to the low toxin concentrations, where many samples were close to the detection limit. Q-PCR analyses revealed the presence of cyanobacteria with the ability to produce microcystin in the pelagic zone at the end of August 2007. Potential microcystin producers, such as *Microcystis aeruginosa*, *Anabaena flos aquae intermedia*, and *Anabaena spiroides*, were present in that station on August 30 and together constituted 1.8% of the total cyanobacterial abundance.

With the exception of 2007, Missisquoi Bay has experienced significant toxic cyanobacterial blooms since 2000. Although we do not have a clear explanation for the absence of a bloom in 2007, we believe that several environmental factors, such as rainfall, the availability of nutrients, and water temperature, may play a role in the bloom dynamics in the bay. Precipitation events for example, especially in the spring following the application of manure and fertilizers, can cause substantial erosion and internal loading of nutrients to the tributaries of Missisquoi Bay and the bay itself. The Quebec-Vermont task force on phosphorus reduction in Missisquoi Bay (9) concluded that 79% of the phosphorus entering the bay is coming from nonpoint sources (erosion and surface runoff) related to agricultural activities. The marked differences in the occurrence of toxic blooms suggest that nutrient inputs alone, however, are not sufficient to explain year-to-year differences. It is hoped that insights into toxigenic strain demographics provided by molecular tools in this and other lakes will provide clues to help us understand and predict *in situ* dynamics.

Conclusions. The occurrence of toxic cyanobacterial blooms is to a large extent unpredictable. Environmental factors, such as temperature, light intensity, and turbidity, as well as nutrient availability, can greatly influence the potency and the extent of blooms. Climatic changes which contribute to intense rainfall episodes and warmer temperatures are making these events even more unpredictable and challenging to understand.

A quantitative PCR (Q-PCR) approach was developed for the early detection of blooms formed by microcystin-producing

cyanobacteria. Oligonucleotide primers for the region of the *mcyD* gene encoding β-ketoacyl synthase were designed and successfully used to characterize cyanobacterial blooms in Missisquoi Bay, Lake Champlain. The Q-PCR method allowed the detection of microcystin-producing cyanobacteria as early as the end of May, before the toxin could be reliably detected by chemical analyses. This result adds to other successes based on molecular approaches that offer new tools to address remaining questions in applied and fundamental cyanobacterial ecology. This technique is promising and can be used for early and efficient monitoring of the most at-risk water bodies.

ACKNOWLEDGMENTS

This research was supported by an NSERC strategic grant and a First Nations and Inuit Health branch grant.

We are very grateful to Serge Paquet for the taxonomy work. We thank Beatrice Barbier for her participation during the DNA extraction optimization phase, Susan Leblanc for the growth and maintenance of *Microcystis* cultures, Hongmei Duan, Frederique Lebrun, and Julie Champagne for their help with filtration, DNA extraction, and Q-PCR, and Jeromy Harvie for toxin analyses.

REFERENCES

1. **Aranda-Rodriguez, R., A. Tillmanns, F. M. Benoit, F. R. Pick, J. Harvie, and L. Solenaia.** 2005. Pressurized liquid extraction of toxins from cyanobacterial cells. Environ. Toxicol. **20:**390–396.
2. **Chorus, I., and J. Bartram (ed.).** 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. World Health Organization. E & FN Spon, London, United Kingdom.
3. **Christiansen, G., J. Fastner, M. Erhard, T. Börner, and E. Dittmann.** 2003. Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. J. Bacteriol. **185:**564–572.
4. **Fewer, D. P., M. Köykkä, K. Halinen, J. Jokela, C. Lyra, and K. Sivonen.** 2009. Culture-independent evidence for the persistent presence and genetic diversity of microcystin-producing *Anabaena* (Cyanobacteria) in the Gulf of Finland. Environ. Microbiol. **11:**855–866.
5. **Findlay, D. L., and H. J. Kling.** 1979. A species list and pictorial reference to the phytoplankton of central and northern Canada, part I. Fisheries and Marine Service manuscript report no. 1503. Western Region Fisheries and Marine Service, Department of Fisheries and the Environment, Winnipeg, Canada.
6. **Fitzgerald, D. J., D. A. Cunliffe, and M. D. Burch.** 1999. Development of health alerts for cyanobacteria related toxins in South Australia. Environ. Toxicol. **14:**203–209.
7. **Harada, K., K. Ogawa, K. Matsuura, H. Murata, M. Suzuki, M. F. Watanabe, Y. Itezono, and N. Nakayama.** 1990. Structural determination of geometrical isomers of microcystins LR and RR from cyanobacteria by two-dimensional NMR spectroscopic techniques. Chem. Res. Toxicol. **3:**473–481.
8. **Harada, K., K. Matsuura, M. Suzuki, M. F. Watanabe, S. Oishi, A. M. Dahlem, V. R. Beasley, and W. W. Carmichael.** 1990. Isolation and characterization of the minor components associated with microcystins LR and RR in the cyanobacterium (blue-green algae). Toxicon **28:**55–64.
9. **Hegman, W., D. Wang, and C. Borer.** 1999. Estimation of Lake Champlain basinwide nonpoint source phosphorus export. Lake Champlain Basin Program Technical Report no. 31. Lake Champlain Basin Program, Grand Isle, VT.
10. **Hisbergues, M., G. Christiansen, L. Rouhiainen, K. Sivonen, and T. Börner.** 2003. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. Arch. Microbiol. **180:**402–410.
11. **Hotto, A. M., M. F. Satchwell, and G. L. Boyer.** 2007. Molecular characterization of potential microcystin-producing cyanobacteria in lake Ontario embayments and nearshore waters. Appl. Environ. Microbiol. **73:**4570–4578.
12. **Jaiswal, P., P. K. Singh, and R. Prasanna.** 2008. Cyanobacterial bioactive molecules—an overview of their toxic properties. Can. J. Microbiol. **54:**701–717.
13. **Jungblut, A.-D., and B. A. Neilan.** 2006. Molecular identification and evolution of the cyclic peptide hepatotoxin microcystin and nodularin, synthetase genes in three orders of cyanobacteria. Arch. Microbiol. **185:**107–114.
14. **Kurmayer, R., G. Christiansen, and I. Chorus.** 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. Appl. Environ. Microbiol. **69:**787–795.
15. **Lyra, C., S. Suomalainen, M. Gugger, C. Vezie, P. Sundman, L. Paulin, and K. Sivonen.** 2001. Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. Int. J. Syst. Evol. Microbiol. **51:**513–526.
16. **Mbedi, S., M. Welker, J. Fastner, and C. Wiedner.** 2005. Variability of the microcystin synthetase gene cluster in the genus *Planktothrix* (Oscillatoriales, Cyanobacteria). FEMS Microbiol. Lett. **245:**299–306.
17. **Nishizawa, T., M. Asayama, K. Fujii, K.-I. Harada, and M. Shirai.** 1999. Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. J. Biochem. **126:**520–529.
18. **Nishizawa, T., A. Ueda, M. Asayama, K. Fujii, K.-I. Harada, K. Ochi, and M. Shirai.** 2000. Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. J. Biochem. **127:**779–789.
19. **Ouellette, A. J. A., S. M. Handy, and S. W. Wilhelm.** 2006. Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molecular characterization of associated cyanobacterial communities. Microbiol. Ecol. **51:**154–165.
20. **Potamis, G. C., M. Papineau, E. Smeltzer, and M. Mimeault.** 2004. International Missisquoi Bay Task Force. Final report to the International Joint Commission, October 20, 2004. International Joint Commission, Ottawa, Canada, and Washington, DC.
21. **Prescotte, G. W.** 1982. Algae of the western Great Lakes area. Otto Koeltz Science Publishers, Koenigstein, Germany.
22. **Rantala, A., D. P. Fewer, M. Hisbergues, L. Rouhiainen, J. Vaitomaa, T. Börner, and K. Sivonen.** 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proc. Natl. Acad. Sci. U. S. A. **101:**568–573.
23. **Rantala, A., P. Rajaniemi-Wacklin, C. Lyra, L. Lepistö, J. Rintala, J. Mankiewicz-Boczek, and K. Sivonen.** 2006. Detection of microcystin-producing cyanobacteria in Finnish lakes with genus-specific microcystin synthetase gene E (*mcyE*) PCR and associations with environmental factors. Appl. Environ. Microbial. **72:**6101–6110.
24. **Rantala, A., E. Rizzi, B. Castiglioni, G. de Bellis, and K. Sivonen.** 2008. Identification of hepatotoxin-producing cyanobacteria by DNA chip. Environ. Microbiol. **10:**653–664.
25. **Rinta-Kanto, J. M., A. J. Ouellette, G. L. Boyer, M. R. Twiss, T. B. Bridgeman, and S. W. Wilhelm.** 2005. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. Environ. Sci. Technol. **39:**4198–4205.
26. **Rinta-Kanto, J. M., E. A. Konopko, J. M. DeBruyn, R. A. Bourbonniere, G. L. Boyer, and S. W. Wilhelm.** 2009. Lake Erie *Microcystis*: relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. Harmful Algae **8:**665–673.
27. **Rosen, B. H., A. Shambaugh, L. Ferber, F. Smith, and M. Watzin.** 2001. Monitoring and evaluation of potential blue green algal toxins in Lake Champlain, summer 2000. Lake Champlain Basin Program, Technical Report no. 39. Lake Champlain Basin Program, Grand Isle, VT.
28. **Rouhiainen, L., T. Vakkilainen, B. L. Siemer, W. Buiema, R. Haselkorn, and K. Sivonen.** 2004. Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. Appl. Environ. Microbiol. **70:**686–692.
29. **Tanabe, Y., K. Kaya, and M. M. Watanabe.** 2004. Evidence for recombination in the microcystin synthetase (*mcy*) genes of toxic cyanobacteria *Microcystis* spp. J. Mol. Evol. **58:**633–641.
30. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
31. **Tillett, D., E. Dittmann, M. Erhard, H. von Döhren, T. Börner, and B. A. Neilan.** 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chem. Biol. **7:**753–764.
32. **Utermöhl, H.** 1958. Zur Vervolkommnung der quantitativen phytoplankton-Methodik. Mitt. Internat. Ver. Für Theoret. Angew. Limnol. **9:**1–38.
33. **Vaitomaa, J. A. Rantala, K. Halinen, L. Rouhiainen, P. Tallberg, L. Mokelke, and K. Sivonen.** 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Appl. Environ. Microbiol. **69:**7289–7297.
34. **Watanabe, M. F., K. I. Harada, K. Matsuura, M. Watanabe, and M. Suzuki.** 1989. Heptapeptide toxin production during the batch culture of two *Microcystis* species (cyanobacteria). J. Appl. Phycol. **1:**161–165.
35. **Welker, M., and H. von Döhren.** 2006. Cyanobacterial peptides—nature's own combinatorial biosynthesis. FEMS Microbiol. Rev. **30:**530–563.